THE UNIVERSITY OF ALBERTA

ANTERIOR MIDGUT PROTEINASE INHIBITOR FROM *GLOSSINA MORSITANS MORSITANS* WESTWOOD (DIPTERA: GLOSSINIDAE) AND ITS EFFECTS UPON TSETSE DIGESTIVE ENZYMES

by

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Abstract

The anterior midgut of *Glossina morsitans morsitans* Westwood contains a proteinase inhibitor, molecular weight 5,500 ± 2,000 daltons, stable to 1 M HCl, heat, and dialysis, but unstable to 1% trichloroacetic acid. Inhibitor activity is not associated with anticcagulant in the anterior midgut. The specific activity of the proteinase inhibitor is similar in mated and unmated females and greater than in male tsetse flies. Proteinase inhibitor inhibits proteinase VI and trypsin hydrolysis of N-benzoyl-L-arginine ethyl ester (BAEE) and benzoyl-DL-arginine-p-nitroanilide (BAPNA) but has no effect on proteinase VI hydrolysis of haemoglobin. Inhibition of trypsin hydrolysis of haemoglobin is noncompetitive. Proteinase inhibitor levels in the anterior midgut decreased immediately after feeding and then increased, reaching a maximum 60-100 hours after ingestion of the bloodmeal. Post-teneral flies contained higher levels of proteinase inhibitor than teneral individuals. Trypsin activity in gut homogenates of *Phormia regina* and *Aedes aegypti* was inhibited. There was no detectable inhibition of bovine or *Pterostichus adstrictus* trypsin activity. Inhibition of *Periplaneta americana* trypsin occurred but was less than fly trypsin inhibition. The possible role of the inhibitor in terminating proteinase production is discussed.

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1. Introduction

1.1 Anterior midgut function in haematophagous insects

The bloodmeal ingested by an insect may darken, indicating digestion, over the entire surface of the meal, as in the mosquitoes, or only in the posterior portion of the meal, as in Glossina morsitans morsitans Westwood. This study is concerned with an insect that shows digestive subdivision of the midgut. Although biochemical and histological studies of the insect digestive system have been done in the past they have failed to distinguish the differences in biochemical function between the anterior and posterior midgut in haematophagous insects.

1.1.1 Biochemical investigations

Studies of the biochemical function of the anterior midgut have tested for digestive enzymes and factors related to blood clotting and fibrinolysis that are involved with ingestion of the bloodmeal. Lester and Lloyd (1928) described an anticoagulant and coagulant from the midgut of Glossina morsitans and Glossina tachinoides. Anticoagulant in the anterior midgut was capable of neutralizing coagulant activity of the posterior midgut and was presumed to be from the salivary gland. The coagulant was likely proteinase present in the posterior midgut (Hawkins 1966). The

anticoagulant function is presumed to insure that ingested blood does not clot while passing through the mouthparts. There appears to be no known function for this factor once the bloodmeal is within the midgut. An anticoagulant and a plasminogen activator have also been detected in whole midgut homogenates of Glossina austeni (Hawkins 1966).

An anticoagulant, Reduvin, was purified from material collected from the punctured abdomen of saline fed Rhodnius prolixus (Markwardt and Schulz 1960). The colourless nature of the material suggested that it originated from the crop, ie. the anterior midgut. The characteristics of Reduvin were similar to Prolixin-G, an anticoagulant isolated from whole midgut homogenates of R. prolixus (Hellmann and Hawkins 1964, 1965). The single difference between Prolixin-G and Reduvin may be related to the differences in the tissues from which the two anticoagulants were purified. There is a plasminogen activator and an inhibitor of fibrinolysis in the whole midgut homogenates of R. prolixus (Hellmann and Hawkins 1965) and Eutriatoma maculatus (Hellmann and Hawkins 1966).

In some bloodfeeding insects part of the bloodmeal remains red after ingestion. This has been observed in Rhodnius prolixus (Wigglesworth 1943), Glossina spp. (Wigglesworth 1929, this study did not include G. m. morsitans), Stomoxys calcitrans (Kuzina 1942), Triatoma infestans, Cimex lecularius (Wigglesworth 1943), and Chrysops silacea (Wigglesworth 1931). Testing the anterior

midgut for enzymes known to occur in the posterior midgut has been negative with R. prolixus (Persaud and Davey 1971), G. m. morsitans (Gooding 1974a, 1977a) and C. silacea (Wigglesworth 1931). Studies of digestive proteinases of S. calcitrans (Champlain and Fisk 1956) and Tabanid flies (Thomas and Gooding 1976; Thomas, Rolseth and Gooding 1976) did not separately test the anterior midgut for proteinase activity. The absence of digestive proteinases from the anterior midgut of these haematophagous insects suggests that this midgut region is not actively involved in hydrolysis of ingested protein. This would be consistent with the function of the region in blood storage. The function of the trypsin inhibitor in the anterior midgut of the tsetse may be to protect the stored bloodmeal and the anterior midgut against proteolysis (Gooding 1974b).

1.1.2 Histological investigations

Most histological investigations have been primarily concerned with the digestive region of the midgut. The anterior midgut in G. m. morsitans has been described at the light microscope (Wigglesworth 1929) and ultrastructural (Boehringer-Schweizer 1977) level and only one cell type is found. In the hungry fly anterior midgut cells are columnar and microvilli at cell apices extend into the lumen. Secretory granules are present and are believed to pass into the lumen. In the teneral fly the region is less differentiated although secretory granules are already

present. In newly engorged flies the cells are flattened and mucous granules are extruded at cell apices. There is an increase in some ultrastructural components after feeding but the amount of mucous secretion remains constant (Boehringer-Schweizer 1977).

Ultrastructural organization of the anterior midgut is consistent with a tissue that must withstand mechanical distension resulting from the large volume of ingested blood. Large numbers of mitochondria present suggest that active transport occurs in this region (Boehringer-Schweizer 1977). Mucilagenous secretions in the anterior midgut may contain trypsin inhibitor or components to facilitate digestive processes in the digestive region of the midgut (Boehringer-Schweizer 1977).

1.1.3 Symbionts

In obligate bloodfeeding insects with a functionally subdivided midgut, symbiotic bacteria are located in the anterior midgut region. In tsetse flies the giant cell zone (Wigglesworth 1929), or mycetome, houses the intracellular symbiont. These bacteroids are retained within the mycetome and do not function in digestion of the bloodmeal (Wigglesworth 1929).

Extracellular symbionts of *R. prolixus* and *Triatoma* spp. are maintained in the anterior midgut between succesive bloodmeals by lodging between the cells of the crop wall. During the digestion of the bloodmeal, symbionts are found

throughout the midgut and hindgut (Goodchild 1955). Rhodnius prolixus maintains a unique relationship with one symbiont, Nocardia rhodnii (Lake and Friend 1967) while in Triatoma spp. a mixture of symbionts may be present (Goodchild 1955; Marchette and Hatie 1965).

It was once thought that symbiont function was to supplement low levels of B vitamins contained within ingested blood (Fraenkel 1952). However recent investigations have shown that in R. prolixus this is not the case (Hill, Campbell and Petrie 1976). The culture of symbiont free G. morsitans (Pell and Southern 1975; Hill, Saunders and Campbell 1973) and R. prolixus (Nyirady 1973; Auden 1974) without serious developmental problems suggests that the symbiont-insect relationship may be other than what was originally thought.

1.2 Digestive proteinases of Glossina morsitans

Tryptase and peptidase activity associated with the posterior midgut of the tsetse fly were first described by Wigglesworth (1929). Tsetse proteinase, with optimal activity at pH 7 to 8, was assayed by Langley (1966) using azocasein as a substrate.

Using a variety of separation techniques and synthetic substrates six alkaline digestive proteinases have been partially purified and characterized from the digestive midgut of G. m. morsitans (Gooding and Rolseth 1976). Trypsin, trypsin-like proteinase VI, chymotrypsin-like

proteinase VII and carboxypeptidase A all exhibit endopeptidase activity. Exopeptidase activity is associated with carboxypeptidase A, carboxypeptidase B and aminopeptidase. All proteinases are extracellular except aminopeptidase which is intracellular. An extracellular haemolytic agent has been detected in the posterior midgut (Gooding 1977b).

1.3 Natural proteinase inhibitors

Natural proteinase inhibitors have been isolated from a variety of plant, animal and microbial sources (see reviews by Vogel et al. 1968; Laskowski and Sealock 1971; Umezawa 1972; Fritz et al. 1974). Natural proteinase inhibitors are polypeptides that primarily inhibit endopeptidases (Vogel et al. 1968). A more rigorous definition of protein proteinase inhibitors as "proteins that may associate reversibly with one or more proteinases to form a complex of discrete stoichiometry in which all the catalytic functions are competitively inhibited," has been proposed (Laskowski and Sealock 1971).

Natural proteinase inhibitors are grouped by molecular weight (Vogel et al. 1968). Low molecular weight proteinase inhibitors, 6,000 to 12,000 daltons, are characteristically acid and heat stable. Included in this group are microbial proteinase inhibitors isolated from culture medium filtrates. Microbial inhibitors have very low molecular weights and may be composed of less than twelve amino acids

(Umezawa 1972). For microbial proteinase inhibitors kinetic analysis is incomplete and evidence suggests that inhibition is competitive or noncompetitive depending upon the substrate and proteinase. Other low molecular weight proteinase inhibitors that have been studied are competitive inhibitors. High molecular weight proteinase inhibitors, 20,000 to 60,000 daltons, are unstable to acid and heat treatment. The group includes plasma, ovomucoid and soybean trypsin inhibitor. Soybean inhibitor, unlike other high molecular weight inhibitors, does not have a carbohydrate component attached to the molecule. All high molecular weight proteinase inhibitors are competitive inhibitors.

1.3.1 Ingested inhibitors of haematophagous insect proteinases

Ingested sera contains at least six inhibitors of mammalian alkaline proteinases (Heimburger 1975) and inhibitors of haematophagous insect proteinases (see reviews by Gooding 1972a, 1975).

Aedes aegypti and *Culex fatigans* trypsin is inhibited by the sera from normal and malarious chicks (Gooding 1966). Seventeen vertebrate sera inhibited partially purified *A. aegypti* trypsin (Huang 1971a) though varied levels of inhibitor were present in different sera. Two serum inhibitors of *A. aegypti* trypsin have been isolated from bovine serum and partially characterized. Both inhibit tryptic hydrolysis of haemoglobin competitively and of

benzoyl-DL-arginine p-nitroanilide (BAPNA) noncompetitively at 37° C (Huang 1971b). Glossina morsitans trypsin is inhibited by two inhibitors from sheep serum (Gooding 1974b) and a serum inhibitor has been partially purified that inhibits Melophagous ovinus trypsin (Gooding 1972b). The kinetics of inhibition by these two proteinase inhibitors are not known.

Honey and lily nectar contain a heat stable inhibitor of mosquito trypsin that may be ascorbic acid and/or riboflavin contained in honey and other carbohydrate food sources. These two vitamins have been shown to inhibit mosquito trypsin (Gooding, Cheung and Rolseth 1973).

1.3.2 Proteinase inhibitors of insect origin

Insect produced proteinase inhibitors have been found in A. aegypti (Yang and Davies 1972), Culex pipiens (Spiro-Kern and Chen 1977), Drosophila melanogaster (Kang and Fuchs 1973, 1974; Kikkawa 1968), Leucophaea maderae Engelmann (1969), Musca domestica (Greenberg and Paretsky 1955), Bulgarian bee venom (Shkenderov 1973, 1975) and G. m. morsitans (Gooding 1974b).

Proteinase inhibitor from bee venom is acid and heat stable with a molecular weight of 9,000 daltons. Inhibition of bovine trypsin hydrolysis of BAPNA is competitive and noncompetitive with casein (Shkenderov 1973,1975). Inhibitors of bovine chymotrypsin and trypsin have been isolated from third instar D. melanogaster (Kang and Fuchs

1973, 1974). The chymotrypsin inhibitor is acid and heat stable with a molecular weight of 12,000 daltons. It has no effect upon bovine, A. aegypti, D. melanogaster or yeast trypsin and does not inhibit activity of larval A. aegypti chymotrypsin-like enzyme (Kang and Fuchs 1974). An inhibitor of A. aegypti chymotrypsin-like enzyme is present in the thorax of adult mosquitoes. It inhibits blackfly chymotrypsin but has no effect upon bovine chymotrypsin. Inhibition of glutaryl-L-phenylalinine p-nitroanilide (GPNA) hydrolysis is competitive (Yang and Davies 1972). The chymotrypsin inhibitor from adult tissue of Culex pipiens has been partially purified and characterized. It is heat stable but unstable to acid and has a molecular weight of 6,800 daltons. It inhibits A. aegypti and C. pipiens chymotrypsin but is not as effective in inhibiting the bovine enzyme. Aedes aegypti trypsin is inhibited by a component of Periplaneta americana haemolymph (Huang 1971a).

A heat stable inhibitor of cockroach alkaline proteinase is located within the gut caeca (Engelmann 1969). Greenberg and Paretsky (1955) found that blowfly gut homogenate had more proteolytic activity than whole fly homogenate suggesting the presence of a proteinase inhibitor in the latter. Gooding (1974b) detected slight inhibition of tsetse trypsin by homogenates of Malpighian tubules, salivary glands and primary excreta from the tsetse. Anterior midgut of unfed flies contained higher levels of inhibitor.

2. Statement of the problem

Vectoring ability of bloodsucking insects has encouraged many authors to study digestion in these animals. Disease causing organisms are usually consumed with the bloodmeal and the first environment encountered by the causitive organism is the alimentary tract. Studies have attempted to elucidate the nature of this environment (see review by Gooding 1972a). In some bloodsucking insects the midgut is functionally subdivided into digestive and nondigestive regions. The nondigestive region has not frequently been studied. Although digestive proteinases are absent from the anterior midgut, the literature contains either little information on active factors in this midgut region and some workers have failed to recognize its differentiation. The present study is an investigation of the trypsin inhibitor in the anterior midgut (Gooding 1974) with the following specific objectives:

1. to determine the nature, source and production of the anterior midgut proteinase inhibitor.
2. to partially purify the proteinase inhibitor and study its interaction with G. m. morsitans digestive proteinases.
3. to examine the interaction of the partially purified inhibitor with trypsin from other animal sources.

3. Materials and methods

3.1 Colony maintainance

Glossina morsitans morsitans Westwood were maintained at 23° C using the method described by Gooding and Rolseth (1976). The insects were fed on rabbits and references to a bloodmeal mean a rabbit bloodmeal.

3.2 Sample preparation

Anterior midgut, without visible trace of blood, was removed from male and/or female *G. m. morsitans*, over a week old, that had starved for 3 to 4 days. Posterior digestive midgut material was collected from male and/or female flies 1 to 3 days after a bloodmeal. Anterior midgut, crop, from *Rhodnius prolixus* Stal, free of blood, was obtained from unfed adult male and/or female insects approximately 25 to 30 days after the adult moult. Posterior digestive midgut material from adult male and/or female *R. prolixus* was collected 5 to 8 days after a bloodmeal. Posterior midgut material was collected for proteinase at or near the time when there was maximum proteinase present.

Posterior midgut was collected from adult female *Phormia regina* 24 hours after being offered chicken liver. The midgut of adult male and/or female

Periplaneta americana, posterior midgut from female Aedes aegypti 24 hours after a bloodmeal, and alimentary tract from Pterostichus adstrictus were also collected. All gut material was stored frozen (-15° C) until required.

Tissue samples were thawed and, unless stated otherwise, homogenized in 50 mM Tris (tris(hydroxymethyl)aminomethane) at 30° C: G. m. morsitans, A. aegypti and P. adstrictus, pH 7.9, P. regina, pH 8.6, P. americana, pH 8.2. Rhodnius prolixus midgut samples were homogenized in 0.1 M potasium phosphate, pH 5.5 containing 3 mM EDTA (ethylenediaminetetraacetic acid). The pH values given are those at which optimal hydrolysis of BAPNA occurs for that particular insect. The pH optimum for Pterostichus melanarius (Gooding and Huang 1969) was used in P. adstrictus trypsin determinations. After centrifugation, 20,000 g for 10 minutes, the supernatant was used in activity determinations.

Partially purified G. m. morsitans digestive proteinases were obtained by the method of Gooding and Rolseth (1976). Bovine trypsin solution was prepared by dissolving pancreatic trypsin, type III, (Sigma Chemical Co.) in 50 mM Tris, pH 7.9 at 30° C, containing 0.05% calcium chloride (Huang 1971a).

3.3 Proteinase determination

For G. m. morsitans proteinase determinations synthetic substrates were prepared as described by Gooding and Rolseth (1976). For trypsin and proteinase VI assays substrates were p-tosyl-L-arginine methyl ester (TAME), N-benzoyl-L-arginine ethyl ester (BAEE) and benzoyl-DL-arginine-p-nitroanilide (BAPNA). For chymotrypsin-like proteinase VII N-acetyl-L-tyrosine ethyl ester (ATEE) was the substrate. For carboxypeptidase A and carboxypeptidase B substrates were hippuryl-DL-phenyllactic acid (HPLA) and hippuryl-L-arginine (HA) respectively. Synthetic substrate benzoyl-DL-arginine naphthylamine (BANA) was prepared for R. prolixus proteinase determinations as described by Houseman (1978). Trypsin activity for gut homogenates from other animal sources was determined by preparing BAPNA using the buffer appropriate for the trypsin source (see 3.2 Sample preparation).

Continuous reaction assays used the substrates BAEE, TAME, ATEE, HA and HPLA. Sample, 0.1 ml, containing proteinase was added to 1 ml of substrate. The absorbance was measured at the beginning and end of the reaction with a Beckman DU-2 spectrophotometer equipped with dual thermospacers and was used for activity determinations. Stop reaction assays were performed with BAPNA and BANA. Proteinase, in 0.5 ml 50 mM Tris, was added to 1 ml BAPNA substrate. The reaction was stopped by adding 0.5 ml of 30% acetic acid. Zero time samples were prepared by adding acid

prior to proteinase. BANA assays were performed as previously described (Houseman 1978).

For tsetse proteinase determinations using synthetic substrates the molar extinction coefficients (see Gooding and Rolseth 1976) were used to determine micromoles of substrate hydrolysed.

Whole protein hydrolysis was determined using a modification of the method of Gooding and Rolseth (1976). Substrate, 0.3 ml 20% denatured haemoglobin in 0.15 M NaCl, was added to 0.3 ml of 0.1 M Tris pH 7.9 at 30° C, containing tsetse proteinase. For R. prolixus protein hydrolysis, casein, 0.3 ml 20% in 0.15 M NaCl, was added to 0.3 ml of 0.1 M potasium phosphate buffer, pH 5.5, containing 20 mM dithiothreitol (DTT) and 40 mM EDTA in which enzyme had preincubated for six minutes. After addition of 0.6 ml trichloroacetic acid (TCA) to stop the reaction, for both assays, samples were spun twice at 15,000 g and absorbance of the supernatant at 280 nm was used for activity determination.

All enzyme assays were carried out at 30° C in duplicate or quadruplicate. For assays using whole protein substrate, controls were run to account for spontaneous breakdown of substrate and endogenous substrate in the gut homogenate. All absorbance measurements were determined using a Beckman DU-2 spectrophotometer equipped with dual thermospacers.

3.4 Inhibitor activity determinations

Proteinase inhibitor activity was determined using a modification of the method described by Huang (1971a). Tsetse proteinase inhibitor and enzyme were combined in 50 mM Tris, pH 7.9 at 30° C, prior to addition of the substrate. For continuous assay reactions proteinase inhibitor and proteinase were combined and diluted to 0.25 ml using 50 mM Tris buffer, pH 7.9 at 30° C, and after incubation for 6 minutes at 30° C 0.1 ml was drawn and added to 1 ml of substrate. For stop reaction assays, proteinase was added to proteinase inhibitor in 0.5 ml of 50 mM Tris for BAPNA or 0.3 ml 0.1 M Tris buffer for haemoglobin assays and incubated for 6 minutes at 30° C prior to addition of the substrate.

Inhibition of proteinase from R. prolixus was determined by examining the effect of R. prolixus crop homogenate upon substrate hydrolysis when added either after DTT activation or prior to DTT activation.

The effect of addition of crop homogenate to thiol activated enzyme was determined for BANA and casein hydrolysis. Crop homogenate was added to preincubation buffer, 1.5 ml of 0.1 M potasium phosphate containing 1.5 mM DTT and 3 mM EDTA, in which proteinase had preincubated for 5 minutes. The substrate, BANA, was added after an additional 6 minutes incubation at 30° C. To determine inhibition of casein hydrolysis, R. prolixus crop homogenate was added to digestive midgut homogenate in 0.1 M potasium

phosphate containing 20 mM DTT and 40 mM EDTA, after 5 minutes incubation at 30° C, to give a total volume of 0.3 ml. This was allowed to incubate an additional 6 minutes at 30° C prior to addition of casein substrate, 0.3 ml 20% in 0.15 M NaCl.

Crop homogenate and enzyme were combined prior to thiol activation and the effect upon BANA hydrolysis was determined. Crop homogenate and digestive midgut homogenate were diluted to 1.4 ml with 0.1 M potasium phosphate buffer, pH 5.5 containing 3 mM EDTA. After 6 minutes at 30° C, 0.1 ml of the same buffer, containing 22.5 mM DTT, was added and allowed to incubate with the enzyme inhibitor solution for 5 minutes at 30° C prior to addition of BANA.

One inhibitor unit (I.U.) is defined as the amount of proteinase inhibitor required to inhibit hydrolysis of one micromole of synthetic substrate per minute.

3.5 Sephadex separations

Anterior midgut used in Sephadex separations was homogenized in the appropriate eluent buffer. Sephadex separations were carried out at 4 to 5° C.

Prelimary experiments were performed, in part, using a small Sephadex G-75 column, 0.65 X 110 cm, equilibrated with 50 mM Tris pH 8.6 at 5° C. Partial purification of the proteinase inhibitor utilized a larger Sephadex G-75 column, 1.6 X 85 cm, equilibrated with the same buffer. A Sephadex G-50 fine column, 0.9 X 104 cm, equilibrated with the same

buffer containing 0.15 M NaCl was utilized for molecular weight determinations and quantification of proteinase inhibitor in anterior midgut homogenates containing blood.

3.6 Determination of proteinase inhibitor in blood protein mixtures using Sephadex G-50

Anterior midgut homogenate was applied to the Sephadex G-50 column and 0.5 ml fractions were collected. The ability of each fraction to inhibit the hydrolysis of BAPNA by a standard digestive midgut homogenate was determined. To determine the number of I.U. present the percent inhibition for those tubes having more than 5% inhibition were summed. The summation was carried out for odd and even numbered fractions and averaged. This was compared to the value obtained by a similar method, using a standard amount of proteinase inhibitor. This technique permits determination of tsetse anterior midgut proteinase inhibitor without interference from ingested serum inhibitors.

3.7 Protein determinations

The method of Lowery *et al.* (1951), using bovine serum albumin as a standard, was used to determine protein.

4. Experiments and discussion

4.1 Preliminary experiments with anterior midgut inhibitor

4.1.1 Preliminary experiments for inhibition of tsetse proteinases

To determine which G. m. morsitans proteinases were inhibited by anterior midgut homogenate, the latter was combined with digestive midgut homogenate and hydrolysis of BAEE, TAME, HA, HPLA, and ATEE determined and compared to digestive midgut homogenate alone (fig. 1). Hydrolysis of TAME and BAEE were inhibited. There was no effect upon hydrolysis of HA or ATEE. Rate of hydrolysis of HPLA decreased but not as markedly as observed with trypsin substrates. Proteinase VI, detected in combination with trypsin in these experiments, is capable of hydrolyzing HPLA (Gooding and Rolseth 1976) and may account for observed inhibition of HPLA hydrolysis by the anterior midgut homogenate. Partially purified carboxypeptidase A was incubated with anterior midgut homogenate and hydrolysis of HPLA determined. There was no detectable inhibition of HPLA hydrolysis (fig. 1).

To determine if proteinase inhibitor was the anticoagulant described from the anterior midgut (Lester and

Lloyd 1928) simultaneous determinations of proteinase inhibitor, anticoagulant, and protein, as absorbance at 280 nm, were carried out from a single fractionation of anterior midgut material using the small Sephadex G-75 column. Salivary gland homogenate was applied to the same column in a second run and assayed for anticoagulant activity (fig. 2). Anticoagulant activity was determined using the partial thromboplastin screening technique (Proctor and Rappaport 1961). Aliqouts of fractions were combined with human test plasma and the assay procedure carried out as described by the authors. Anterior midgut proteinase inhibitor is eluted independently of anticoagulant activity. Midgut anticoagulant is not eluted from the column at the same point as salivary anticoagulant (fig. 2).

To determine if proteinase inhibitor in the anterior midgut was from ingested serum, rabbit serum was applied to the large Sephadex G-75 column and ability of collected fractions to inhibit BAPNA hydrolysis by digestive midgut material was determined and compared to fractionated anterior midgut. No inhibitor with a molecular weight similar to tsetse inhibitor was eluted from the column (fig. 3). Characteristically proteinase inhibitors in serum are associated with the high molecular weight components (Vogel et al. 1968).

Specific activity and amount of proteinase inhibitor present in anterior midgut of males and mated and unmated females all starved 4 days after feeding were determined by

inhibition of BAPNA hydrolysis by digestive midgut homogenate (table 1). Highest levels of inhibitor were found in mated females and the lowest levels in males. Specific activity of the inhibitor in mated and unmated females was similar and this was greater than inhibitor in male tsetse flies.

4.1.2 Partial purification of the inhibitor

Anterior midguts from starved male and/or female flies were collected and homogenized. The material, after centrifugation, was fractionated using the large Sephadex G-75 column. Collected fractions were assayed for tryptic hydrolysis of BAPNA, inhibition of BAPNA hydrolysis by digestive midgut material, and protein, as absorbance at 280 nm (fig. 4). The peak of inhibitor activity was pooled and concentrated using Aquacide II (Calbiochem). The concentrate was dialyzed, at 5° C, against 50 mM Tris, pH 8.6 at 5° C, and a final 5.3 fold purification was achieved (table 2).

4.1.3 Studies with partially purified inhibitor

Partially purified proteinase inhibitor was combined with tsetse trypsin and proteinase VI. Hydrolysis of BAEE, BAPNA and haemoglobin was determined. Proteinase VI hydrolysis of BAEE and BAPNA was inhibited but there was no detectable inhibition of haemoglobin hydrolysis (fig. 5a). Decrease in trypsin hydrolysis was similar for the three substrates (fig. 5b).

4.1.4 Discussion

Preliminary results suggest that anterior midgut proteinase inhibitor inhibits primarily the activity of trypsin and/or proteinase VI (fig. 1). Further experiments were concerned with the interaction of anterior midgut proteinase inhibitor with these two proteinases.

Higher levels of proteinase inhibitor in female flies (table 1) are similar to findings that digestive processes occur at elevated levels in female bloodsucking insects (see review by Gooding 1972a). Tsetse females ingest larger bloodmeals and have higher levels of digestive proteinases than males (Gooding 1977a).

Previous studies showed anterior midgut inhibitor increased in specific activity after ingestion of the bloodmeal. It was not determined whether the increase resulted from serum inhibitors being selectively retained or from production of an inhibitor of fly origin (Gooding 1974b). The inhibitor in the anterior midgut homogenate has a lower molecular weight than the inhibitors in serum (fig. 3). The increase in specific activity after feeding could be the result of proteinase inhibitor production by the fly. It has been suggested that the function of such an inhibitor was to protect the anterior section against damage by the posterior midgut trypsin (Gooding 1974b). Inhibition of only trypsin and/or proteinase VI would appear to not be effective in protecting the anterior midgut or the stored

bloodmeal frcm premature digestion by carboxypeptidase A, carboxypeptidase B and proteinase VII.

Anticoagulant activity was eluted from the Sephadex G-75 column independently of proteinase inhibitor activity (fig. 2). The posterior midgut coagulant inhibition by anterior midgut anticoagulant reported by Lester and Lloyd (1928) may have been anterior midgut inhibition of digestive midgut proteinase. Early studies of haematolcgical factors in the tsetse fly failed to distinguish differences between blood clotting factors and possible similar effects that may have resulted from digestive enzymes and related compounds within the midgut.

Anticoagulant in the anterior midgut had a higher molecular weight than salivary anticoagulant, based upon observed elutien profiles (fig. 2). This suggests that, as in R. prolixus, there is a separate salivary and midgut anticoagulant. It was not possible from these experiments to preclude the possibility that differences in molecular weight resulted from combination of salivary gland inhibitor with proteins contained in the anterior midgut.

Elution of proteinase inhibitor independent of the interstitial volume of the column suggests that B vitamins are not responsible for inhibition of digestive proteinases in a manner similar to the interaction of mosquito trypsin and ascorbic acid (Gooding et al. 1973).

4.2 Characteristics of the inhibitor

4.2.1 Molecular weight determination

Molecular weight of partially purified proteinase inhibitor was determined using the Sephadex G-50 column calibrated for molecular weight determination, according to the method of Andrews (1964), using trypsin (24,000) cytochrome C (12,400) myoglobin (17,800) and pancreatic trypsin inhibitor (6,500). A straight line was fitted to the calibration points using linear regression. The molecular weight of the proteinase inhibitor was 5,500 ± 2,000 daltons (fig. 6). The standard deviation was obtained by applying the standard deviation of the line to the molecular weight.

4.2.2 Stability to acid, heat and dialysis

Anterior midgut homogenate, 0.018 mg protein, was diluted with 50 mM Tris, pH 7.9 at 30° C, to a volume of 0.5 ml and heated, 80° C, for various lengths of time. After heat treatment samples were placed on ice. After equilibration to 30° C the ability to inhibit hydrolysis of BAPNA by digestive midgut homogenate was determined. Anterior midgut material that had been heated was found to have the same capacity to inhibit hydrolysis of BAPNA as unheated material (fig. 7).

Anterior midgut material, 0.018 mg protein, in 0.02 ml of 50 mM Tris pH 7.9 at 30° C, was diluted to 0.4 ml with 1 M HCl and incubated for various lengths of time at 30° C. The pH was adjusted to 7.9 by adding 0.4 ml of a solution, pH unknown, made up of 1.5 M Tris, pH 7.9 at 30° C to which was added NaOH to a final concentration of 1 M. The ability of acid treated material to inhibit hydrolysis of BAPNA by digestive midgut material was compared to anterior midgut material added to previously neutralized acid. Stability of the anterior midgut inhibitor to 1% TCA was determined in a similar manner using 1% NaOH to neutralize the acid. Proteinase inhibitor treated with HCl had the same capacity to inhibit BAPNA hydrolysis as proteinase inhibitor added to the neutralized HCl mixture. Treatment with TCA decreased the proteinase inhibitor capacity to inhibit hydrolysis of BAPNA by digestive midgut homogenate. (fig. 7).

Stability to dialysis was determined by dialyzing, using cellulose dialysis tubing, anterior midgut inhibitor against over 200 times the volume of 50 mM Tris pH 8.5 overnight at 5° C. The material contained 0.118 I.U./ml before and after dialysis. Inhibition of BAPNA hydrolysis by digestive midgut homogenate was used to determine the I.U.'s present.

4.2.3 Kinetics of inhibition

Inhibition kinetics were determined with partially purified proteinase inhibitor and tsetse trypsin. Data were plotted using the double reciprocal plot and linear regression of the points determined (fig. 8). Proteinase inhibitor is a noncompetitive inhibitor of tsetse trypsin. Observed Km values (table 3) are in agreement with those previously reported (Gooding and Rolseth 1976).

4.2.4 Discussion

Variation observed in molecular weight results from problems in calibrating for low molecular weight determinations. High molecular weight standards maintain good linear relationship but pancreatic trypsin inhibitor is not eluted in the same way. Variation in molecular weight made it impossible to determine whether tsetse inhibitor is similar to proteinase inhibitors from microbial sources.

Heat and HCl stability are characteristic of other low molecular weight proteinase inhibitors (Vogel et al. 1968). A common step in purification of low molecular weight proteinase inhibitors uses TCA precipitation. The TCA instability of tsetse proteinase inhibitor is not a common characteristic of low molecular weight proteinase inhibitors. Denaturing effects of TCA must irreversibly disrupt the inhibitor in a manner differing from simple HCl treatment. Usually charged molecules or those with a molecular weight greater than 10,000 daltons will not pass

through dialysis membrane (Freifelder 1976). Specialized tertiary or quaternary organization, molecular charge or aggregation of the inhibitor molecule may account for the inability of the compound to pass through cellulose dialysis membrane.

Noncompetitive inhibition is not characteristic of protein proteinase inhibitors (Laskowski and Sealock 1971). Bee venom noncompetitively inhibited bovine trypsin hydrolysis of casein (Shkenderov 1975). Bovine serum inhibited BAPNA hydrolysis by A aegypti trypsin noncompetitively at 37° C (Huang 1971b). For other inhibitors of insect origin kinetics analysis is not available. The noncompetitive inhibition observed in these two studies may be related to species differences between the inhibitor and the proteinase.

4.3 Production of the inhibitor

4.3.1 Localization of the inhibitor

Anterior midgut wall and lumen content from 21 post-teneral female flies 48 to 56 hours after feeding were collected using the method described by Gooding (1977b). Material was homogenized in 50 mM Tris 0.15 M NaCl buffer and 1.44 mg of lumen or 0.445 mg of anterior midgut wall protein was applied to the Sephadex G-50 column in a volume of 0.5 ml. Proteinase inhibitor levels were determined using

the Sephadex G-50 separation technique. The lumen contained 0.013 I.U. per anterior midgut compared to the wall material that contained 0.001 I.U. per anterior midgut. This represents a 13 X greater amount of the inhibitor within the lumen compared to the gut wall.

4.3.2 Production of the inhibitor in teneral and post-teneral flies

Anterior midgut from 3 day old teneral flies and two week old post-teneral female flies were collected in batches of 10-15 from flies prior to feeding or from flies at various times after ingestion of a bloodmeal and homogenized in 50 mM Tris 0.15 M NaCl buffer. Protein content was determined for each sample. Proteinase inhibitor determinations were carried out by applying sample homogenates, in a constant volume of 50 mM Tris 0.15 M NaCl, to the Sephadex G-50 column. There was more protein in the anterior midgut of teneral flies than in the anterior midgut of starved post-tenerals (fig. 9a). The statistical significance of this was not determined. The rate of decline after ingestion was similar in both. Initial proteinase inhibitor levels were higher in post-teneral flies. In both, proteinase inhibitor level dropped after feeding and rose reaching a maximum 60 to 100 hours after ingestion of the bloodmeal. The maximum proteinase inhibitor levels in teneral individuals were higher than the level observed in unfed tenerals. Proteinase inhibitor reached levels similar

tc those at initial feeding in post-teneral flies (fig. 9b).

Experiments were done to determine whether the decrease in proteinase inhibitor immediately after feeding resulted from adsorption or inactivation of the proteinase inhibitor by ingested blood material. Crop homogenate was prepared by homogenizing blood filled crop from newly fed flies in 50 mM Tris buffer, containing 0.15 M NaCl. The equivalent of 7.2 flies was combined with anterior midgut material from the equivalent of 3.3 flies and incubated at 30° C for 15 minutes prior to application to the Sephadex G-50 column. Collected fractions were assayed for inhibitcr, and proteinase inhibitor levels were ccmpared to fractionation of a similar quantity of proteinase inhibitor in buffered saline. Crop-proteinase inhibitor mixture contained 0.038 I.U. while proteinase inhibitor and buffered saline contained 0.040 I.U. There appears to be no detectable adsorption or deactivation of proteinase inhibitor by the ingested meal.

To determine if proteinase inhibitor is passed into the digestive midgut the posterior midgut was removed, 3 or 8 hours after feeding, from post-teneral female flies that had a single blocdmeal, after starving for 3 to 4 days, and homogenized in 50 mM Tris 0.15 M NaCl buffer. Homogenate, containing the equivalent of 5 digestive midguts, was applied to Sephadex G-50 for detection of proteinase inhibitor. Nc proteinase inhibitor was found in the posterior midgut.

4.3.3 Discussion

The location of inhibitor within the midgut lumen and no apparent deactivation or adsorption of inhibitor by ingested blood suggested that decreased inhibitor levels in the anterior midgut after feeding result from the inhibitor being passed back into the digestive midgut. However, inhibitor was not found in the posterior midgut. This probably resulted from combination of large amounts of trypsin with proteinase inhibitor. Compartmentalization or special spacial distribution of inhibitor within the digestive midgut would have been disrupted with homogenization resulting in no detection of unbound proteinase inhibitor. It was not determined whether proteinase inhibitor was mixed with the bloodmeal or was passed back in combination with the peritrophic membrane.

Proteinase inhibitor production (fig. 9b) suggested a cyclic production of the inhibitor that may be controlled by a factor related to feeding. This study did not elucidate anything concerning the nature of the control mechanism involved.

Proteinase inhibitor in post-teneral flies did not reach levels that corresponded to those at initial feeding. This may have resulted from differences in the time since the last meal of flies used for initial proteinase inhibitor determinations or may be the result of starvation prior to feeding.

Boehringer-Schweizer (1977) suggested that the mucous secretion in the anterior midgut contained the trypsin inhibitor detected by Gooding (1974b). However, the mucous content in the anterior midgut is constant throughout the hunger cycle, this does not follow the cyclic pattern shown by proteinase inhibitor content. It is not known if the mucous secretion has a constant composition throughout the feeding cycle of the tsetse fly.

The pattern of change in proteinase inhibitor after feeding may explain observations (Lester and Lloyd 1928) used as evidence that anticoagulant within the anterior midgut was from the salivary glands. Flies 24 hours after feeding, with salivary glands removed, had decreased levels of anticoagulant within the anterior midgut compared to "normal" flies. The age of "normal" flies was not given and a control to indicate if anticoagulant levels were compared to unoperated flies 24 hours after feeding, was not performed. Lower anticoagulant levels may have been brought about in a manner similar to decreased proteinase inhibitor levels observed in these experiments. Decreased levels do not preclude the possibility of anterior midgut anticoagulant in the tsetse fly.

4.4 Inhibition of trypsin from other species and proteinase from Rhodnius prolixus

Crude gut homogenate from *P. regina*, *P. americana*, *P. adstrictus*, *A. aegypti* and *G. m. morsitans* and a solution of

bovine trypsin was added to varied amounts of partially purified proteinase inhibitor and the hydrolysis of BAPNA was determined. Trypsin activity from A. aegypti, G. m. morsitans and P. regina was inhibted to a similar extent. Bovine and P. adstrictus trypsin were not detectably affected. Periplaneta americana trypsin was inhibited but not to the extent that the fly trypsins were affected (fig. 10).

Rhodnius prolixus crop homogenate inhibition of BANA and casein hydrolysis by digestive midgut homogenate proteinase activity from R. prolixus was examined. Inhibition of casein hydrolysis was determined by combining the thiol activated enzyme with crop homogenate as previously outlined. With R. prolixus hydrolysis of BANA and casein were inhibited by addition of crop homogenate (fig. 11).

4.4.1 Discussion

It was not possible to determine whether observed inhibition groupings (fig. 10) were related to taxonomic or ecological considerations. The experiments were performed to determine whether inhibition of tsetse trypsin was unique, possibly giving an implication of symbiont function as this in itself is a unique relationship.

Trypsins from these insects are not all well studied and their characteristics are not fully understood. Use of gut homogenates did not permit detection of a simple enzyme

inhibitor system. Gut homogenate may contain a variety of BAPNA hydrolyzing enzymes, other inhibitors or compounds protecting against the inhibition of activity. The interaction and effect of the tsetse inhibitor on trypsin in the gut homogenates that has been observed can not be discussed in relation to the interaction of proteinase and inhibitor but only with regard to the digestive abilities of the gut.

Differences in observed inhibition illustrates the importance of using proteinases from the same animal from which proteinase inhibitor was isolated. Only a few insect produced proteinase inhibitors have been detected using insect proteinase. In studies of mammalian proteinases and proteinase inhibitor there are species differences related to the nature of the inhibitor (Greene et al. 1974). Some authors have suggested that inhibition that has been reported in the literature using mammalian sera and bovine enzymes are test tube phenomenon and do not relate to results that are obtained when human enzymes and human pancreatic inhibitor are studied (Jeppson and Laurel 1974; Beith and Aubrey 1974). This same warning applies to inhibition of insect proteinases. For results to be meaningful proteinase and proteinase inhibitor being examined must interact with each other under natural conditions. It is possible that many previous tests resulting in no inhibition when non-insect enzymes were examined may be artifacts and if insect proteinases were

used then inhibition may have been detected.

With R. prolixus (fig. 11) considerably better inhibition was observed with casein substrate than BANA. Clouding occurred in the BANA assays when more than reported amounts of crop material was added to the assay mixture. In these results there may still be some effect from excess amounts of protein in the assay mixture that created differences in the observed extent of inhibition between BANA and casein substrates.

Results with R. prolixus do not indicate whether the crop contains a unique proteinase inhibitor. Inhibition may have resulted from the effect of Prolixin-G. This does indicate a possible similarity in the action of the midgut in these two insects. In R. prolixus considerably more crop protein is required to bring about inhibition and this may be related to differences in proteinase or the alimentary system in the two insects.

5. General Discussion

5.1 Nature of the inhibitor in Glossina morsitans

Proteinase inhibitor in the anterior midgut of the tsetse fly inhibits BAPNA, BAEE and haemoglobin hydrolysis by tsetse trypsin. Hydrolysis of BAPNA and BAEE by proteinase VI is inhibited but hydrolysis of haemoglobin is not affected. Proteinase inhibitor activity has been shown to be independent of anticoagulant activity associated with the anterior midgut.

The inhibitor resembles other low molecular weight proteinase inhibitors in heat, HCl stability and molecular weight. Noncompetitive inhibition is a property of the anterior midgut inhibitor from the tsetse, but not characteristic of other naturally occurring proteinase inhibitors.

Two reports of noncompetitive inhibition relate to insect studies. Mosquito trypsin hydrolysis of BAPNA is noncompetitively inhibited by two serum inhibitors at 37° C (Huang 1971b) and bee venom is a noncompetitive inhibtor of casein hydrolysis by bovine trypsin (Shkenderov 1975). Noncompetitive inhibition may be related to species differences between inhibitor and proteinase. The present study described the interaction of tsetse proteinase inhibitor and proteinase from a single animal source.

Studies of mammalian proteinase and mammalian inhibitor interactions have shown only competitive inhibition (Laskowski and Sealock 1971).

During the study no attempt was made to distinguish differences between anterior midgut and the mycetome. These investigations do not exclude the possibility that the symbionts produce the proteinase inhibitor.

5.2 Possible role of the inhibitor in Glossina morsitans

In general, proteinase inhibitors are believed to function by "limiting the area affected by proteolytic processes as well as the duration of the processes" (Vogel et al. 1968). Plasma inhibitors are believed to protect the plasma proteins from proteolysis (Beith and Aubrey 1974) and pancreatic inhibitors are presumed to insure that the protease zymogens in the pancreas are not prematurely activated causing damage to pancreatic tissues (Feinstein et al. 1974). It is possible that proteinase inhibitor in the midgut of the tsetse could have a function similar to that of mammalian inhibitors. Experiments have shown that tsetse proteinase inhibitor is not effective in inhibiting all proteinases that occur in the digestive tract of the fly. Therefore, the inhibitor would not provide complete protection of the midgut wall or stored bloodmeal from proteolysis unless trypsin attack precedes all other proteolytic activity.

Protection could occur if inhibition of trypsin resulted in a decrease in action of the other proteinases. Trypsin and carboxypeptidase B are correlated in the tsetse fly (Gooding 1977c). Trypsin produces peptides that have the specifity requirements of carboxypeptidase B. A similar relationship with proteinase VII and carboxypeptidase A is possible but has not been demonstrated. Control of trypsin and in turn other proteinases would require that as a result of some action by trypsin, a factor is produced that affects production of other proteinases. For the proteinase inhibitor to protect against proteolysis by all the digestive proteinases would require that trypsin production is affected resulting in the decrease in other proteinases.

It is difficult to attribute a classical proteinase inhibitor role, of protection from proteolysis, to the inhibitor in the anterior midgut of the tsetse fly. Location of inhibitor within the midgut lumen, its cyclic nature of production related to feeding, higher levels in female flies compared to males, and the inhibitory effects upon two digestive proteinases suggest a digestive role for the tsetse proteinase inhibitor.

Noncompetitive inhibition by proteinase inhibitor of haemoglobin hydrolysis by trypsin and inhibition of synthetic substrate hydrolysis by trypsin and proteinase VI, suggest that inhibitor is not involved in active site binding with these two enzymes. In the case of trypsin inhibition of haemoglobin hydrolysis, this conclusion is

consistent with the type of inhibition. For proteinase VI the conclusicn is inferred from available data. Active site binding of an inhibitor most frequently creates steric hindrance or blocking that prevents substrate binding. Enzyme substrate interaction for binding whole protein substrate may be affected while binding of synthetic substrates may not be altered causing inhibitiocn of whole protein hydrclysis and no inhibition of esterase activity. The opposite has been reported with proteinase VI. If inhibitor should bind at a site other than the active site, conformaticnal changes may alter the binding of only small synthetic substrates to enzyme, while complex interactions required to bind large protein molecules may not be affected. This could result in an alteration of the synthetic substrate hydrolysis but not haemoglobin proteolysis.

Proteinase VI and trypsin activity are inhibited by interaction of proteinase inhibitor and prcteinase. This suggests that the inhibition observed may be secondary to a more fundamental role that is related to binding interactions of inhibitor with proteinases rather than inhibitiocn. Tests with partially purified enzymes were not done for interaction between proteinase inhibitor and those proteinases that, in preliminary investigations, were not inhibited.

Proteinase inhibitor may be involved in shutting down digestive proteinase production. Highest levels of the

inhibitor occur when proteinases present within the digestive midgut are decreasing. It has been postulated that a single operon, containing the structural genes for tsetse proteinases, is responsible for tsetse digestive enzyme production (Gooding 1977c). The operon may be controlled through a regulator produced in response to the proposed ingested secretagogue. The operon in turn may be repressed, through a repressor produced in response to the interaction of trypsin, or other proteinases, with proteinase inhibitor from the anterior midgut. This would limit or control the duration of proteolysis within the midgut. The proteinase inhibitor may be contained within the lumen or on the peritrophic membrane. The location of this factor on peritrophic membrane would insure that the stimulant for the repressor would be located adjacent to the midgut epithelial cells thus facilitating its action on these cells.

In insects the peritrophic membrane is a unique structure that has been described as a noncellular membrane selectively permeable to high molecular weight complexes. (Zhuzhikov 1964,1970). The peritrophic membrane of Musca domestica permits human amylase to pass from the exterior into the interior of the peritrophic membrane but not vice versa (Zhuzhikov 1964). If the tsetse peritrophic membrane functions in a manner similar to that observed in M. domestica then there must be a factor in the peritrophic membrane system that recognizes proteinases in order to insure unidirectional movement. Such an interaction would

involve some form of binding to the enzyme.

The recognition factor may be proteinase inhibitor that has been detected in these studies. Peritrophic membrane production occurs in the proventriculus (Wiggelsworth 1929; Moloo et al. 1970). Information is not available to determine whether other compounds are added to the peritrophic membrane once within the anterior midgut region. Chemical maturation or modification of the peritrophic membrane as it passes through the anterior midgut may involve addition of the detected inhibitor.

If proteinase inhibitor in these studies is responsible for insuring unidirectional movement of proteinase through the peritrophic membrane then the presence of the factor would be expected to occur at the same time as the maximum of proteinase at 24 to 48 hours after ingestion of the meal (Gooding 1977a). This does not occur.

If the secretagogue in the tsetse is ingested trypsin inhibitor (Gooding 1977c), and if the proteinase inhibitor of tsetse origin also serves as a secretagogue, it is possible to explain production of a proteinase inhibitor by the tsetse as a mechanism to insure rapid digestion of the blood meal. Extra secretagogue added by the anterior midgut may insure that sufficient levels of proteinase are produced. It has been suggested that Tabanids use a blood serum activator to insure a quick and rapid proteolysis of ingested meal (Gooding, Rolseth and Thomas 1977). A similar situation may occur in the tsetse with increased proteinase

as a result of increased secretagogue levels.

Proteinase inhibitor in the anterior midgut reaches a maximum 60-100 hours after ingestion of the bloodmeal. Maximum proteinase (Gooding 1977a) and haemolysin (Gooding 1977b) occur 24-48 hours after ingestion of the bloodmeal. The peak in proteinase and haemolysin does not correspond to that of proteinase inhibitor. The largest amounts of proteinase inhibitor are present in the anterior midgut when small amounts of the bloodmeal are still present and yet to be passed back to the digestive midgut. The movement of this final material into the digestive region would, according to this hypothesis, cause an increase in proteinase just prior to starvation. This has not been observed in G. m. morsitans.

5.3 Suggested future research

Present knowledge of the function of the peritrophic membrane is limited (see review by Richards and Richards 1977). In the tsetse fly information about the peritrophic membrane concerns production in the teneral fly (Molco et al. 1970). Production of the peritrophic membrane after feeding and during the hunger cycle is not understood.

To determine whether proteinase inhibitor in this study was responsible for the binding of the digestive proteinases would require further work. Changes in kinetic parameters of the proteinase and physical biochemical characteristsics need to be examined for possible interaction for all tsetse

proteinases with inhibitor. Experiments on production of the peritrophic membrane and its subsequent possible modificaticns once produced need to be carried out.

Further experiments are required to provide evidence that a single operon is responsible for proteinase production. An examination of the correlation between the production of all the tsetse proteinases should be carried out in order to support the single operon hypothesis. If it is suspected that ingested proteinase inhibitor is responsible fcr induction of the operon then feeding experiments using serum proteinase inhibitcrs should be performed to test this. Feeding experiments using the inhibitor-proteinase complex would be of value in determining if the interaction of these two components is responsible for repression of the tsetse proteinase operon.

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Table 1. Proteinase inhibitor in the anterior midgut of mated and unmated female and male tsetse flies starved 4 days.

	I.U./ gut	I.U. / mg of gut protein
unmated females	0.006	0.211
mated females	0.009	0.236
males	0.003	0.067

Table 2. Overall purification of the anterior midgut proteinase inhibitor.

Specific activity (I.U. / mg protein)			
Crude homogenate	G-50 Pool after Aquacide II	Pool after dialysis	Purification[a]
0.218	1.022	1.146	5.3

a purification is the ratio of the specific activity of the pool after dialysis to crude homogenate.

Table 3. Effect of substrate concentration and proteinase inhibitor on trypsin hydrolysis of haemoglobin.[a]

	Km (mg / ml)	95% confidence limits	
Trypsin (Gooding and Rolseth 1976).	3.43	2.87	4.15
Trypsin with no inhibitor.	4.1	2.3	5.8
Trypsin with 0.006 mg inhibitor	3.5	2.3	5.8
Trypsin with 0.009 mg inhibitor	4.0[b]		

a Data from figure 8.
b Based on two points only

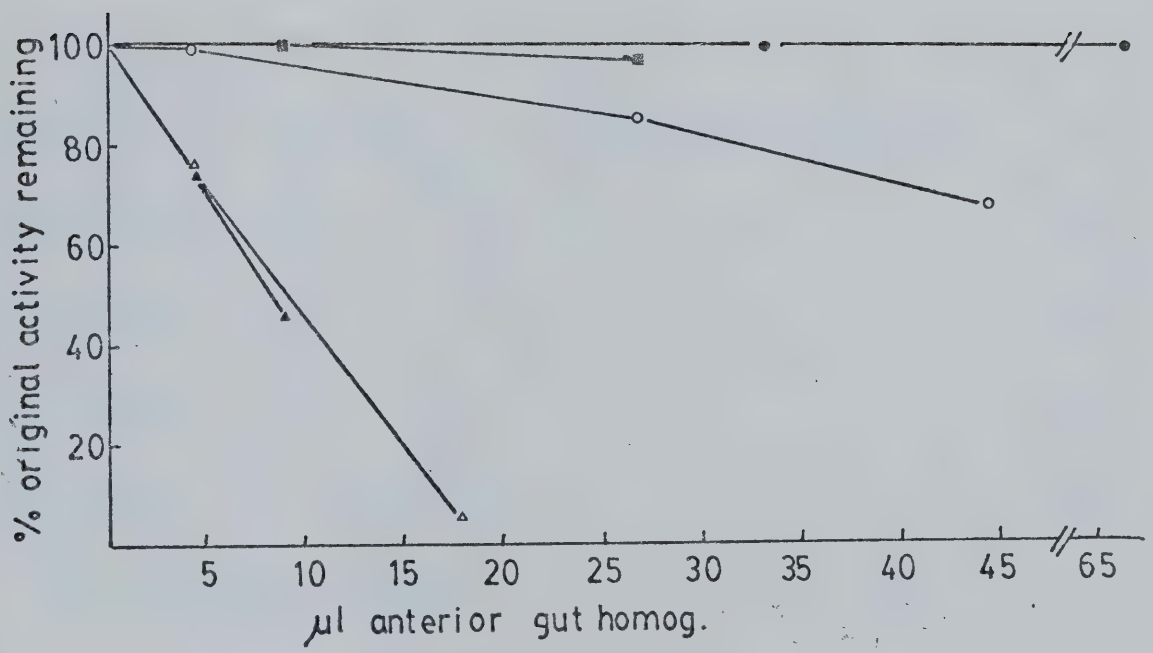

Figure 1. Activities of tsetse proteinases, in digestive midgut homogenate, and partially purified carboxypeptidase A in the presence of anterior midgut homogenate (0.12 mg protein/ml). 100% activities, as micromoles of substrate hydrolyzed per minute, and principal proteinases that hydrolyze the substrates are given as follows: BAEE, trypsin and proteinase VI, 0.0282 micromoles (△—△), TAME, trypsin and proteinase VI, 0.0555 micromoles (▲—▲), HPLA, carboxypeptidase A, 0.0123 micromoles (○—○), HA, carboxypeptidase B, 0.0125 micromoles (■—■), ATEE, proteinase VII, 0.0023 micromoles (■—■), and HPLA, by partially purified carboxypeptidase A, 0.0061 micromoles (●—●).

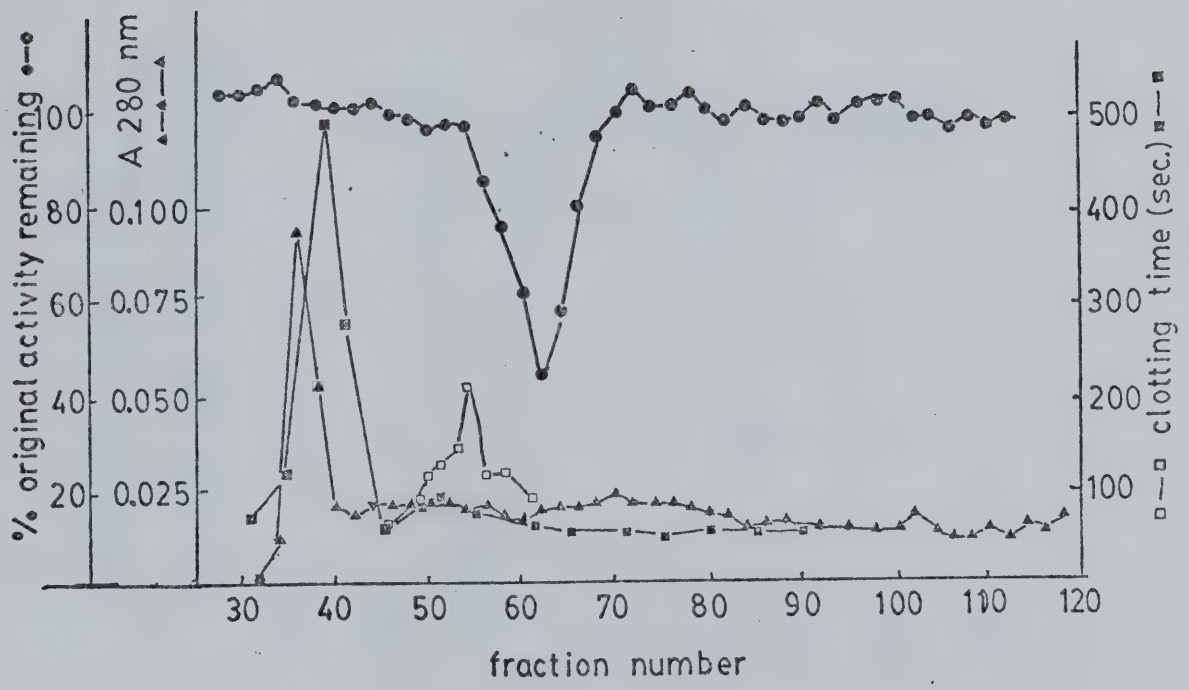

Figure 2. Sephadex G-75 separation of tsetse starved anterior midgut and salivary gland homogenates. For anterior midgut fractionation 100% tryptic activity is hydrolysis of 0.0150 micromoles of BAPNA per minute (● — ●), and protein (▲ — ▲) as absorbance at 280 nm. Anticoagulant of salivary gland (□ — □) and anterior midgut (■ — ■) was determined by adding 0.1 ml of fraction to human test plasma and assayed using the partial thromboplastin screening technique (Proctor and Rapaport 1961).

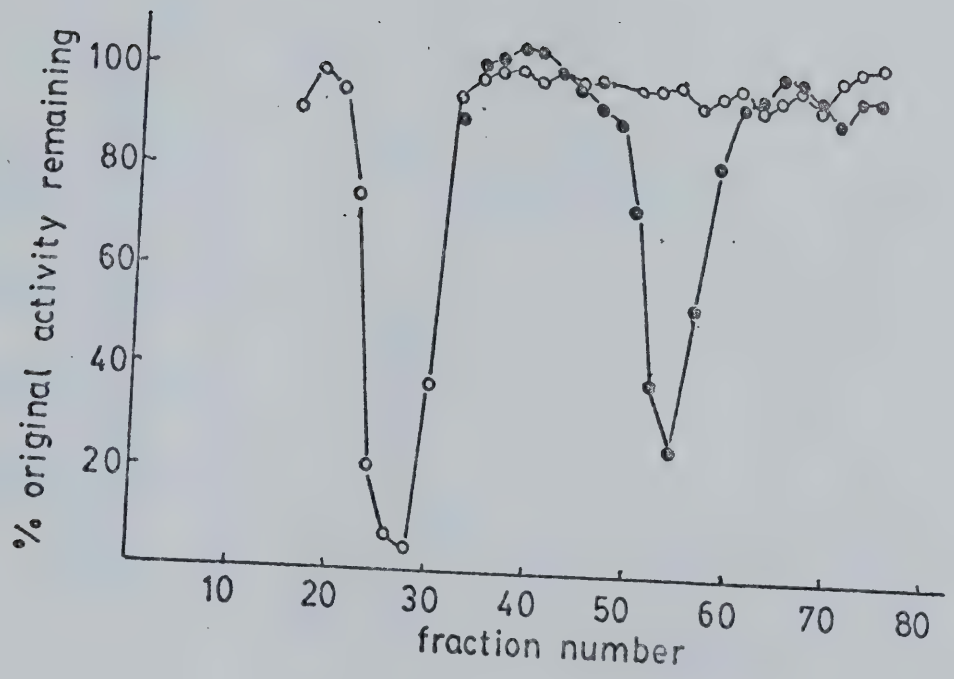

Figure 3. Inhibition of tsetse digestive midgut hydrolysis of BAPNA by tsetse anterior midgut homogenate and rabbit serum fractionated using Sephadex G-75 column. 100% activities as micromoles of BAPNA hydrolysed per minute as follows; 0.0087 micromoles for serum (○ — ○) and 0.0124 micromoles for anterior midgut (● — ●).

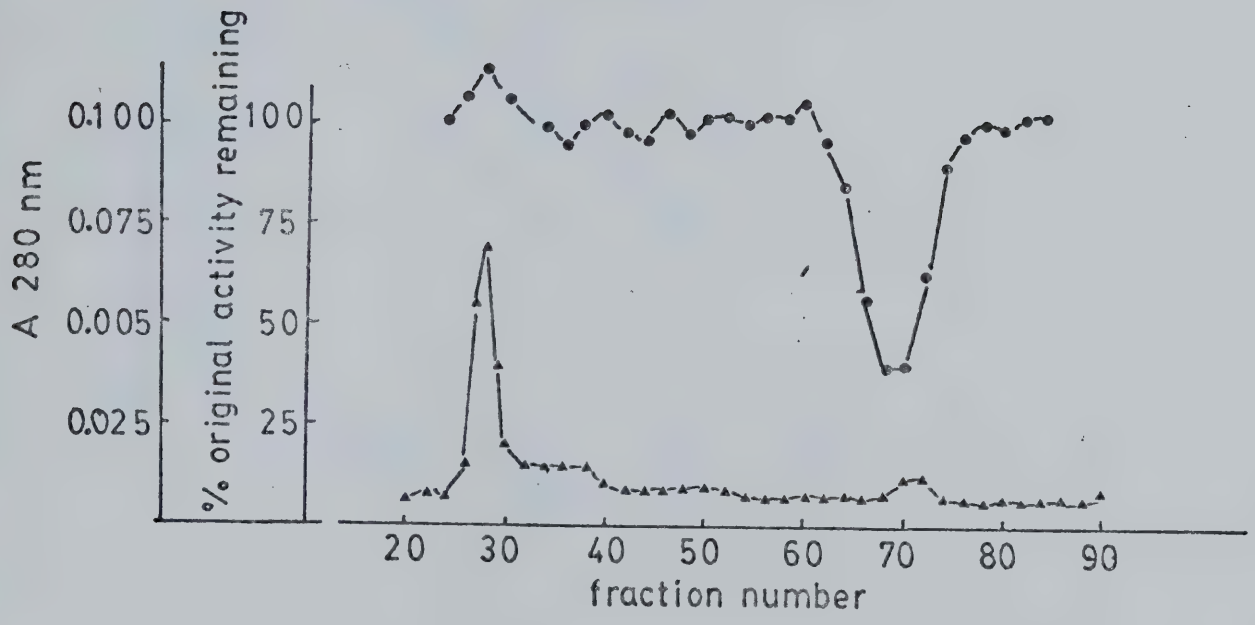

Figure 4. Sephadex G-75 separation of anterior midgut homogenate in partial purification of tsetse proteinase inhibitor 100% tryptic activity (● —●) is hydrolysis of 0.0051 micromoles of BAPNA per minute. Protein (▲—▲) as absorbance at 280 nm. No tryptic activity was present in the collected fractions.

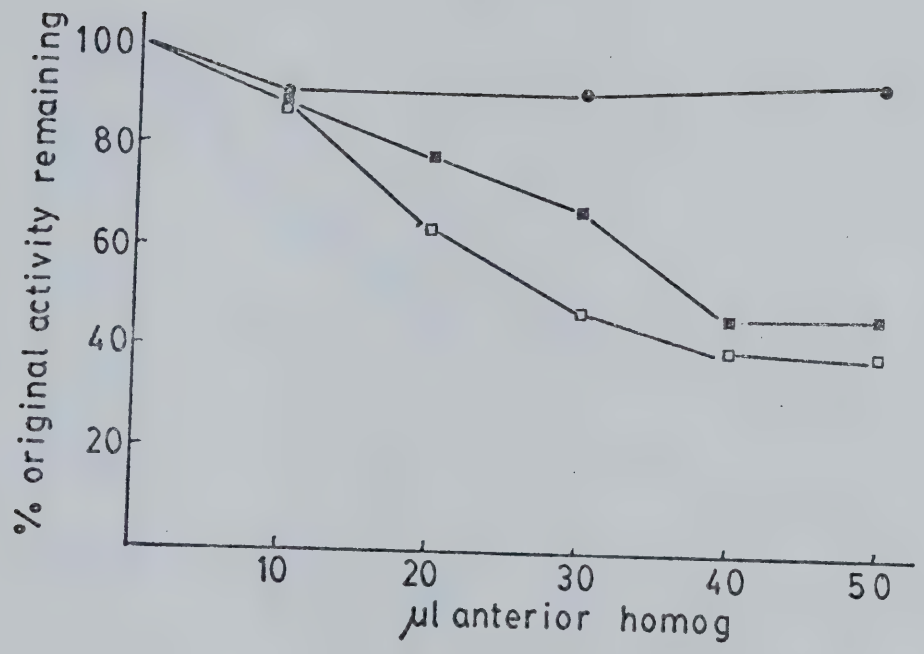

Figure 5a. Inhibition of tsetse proteinase VI hydrolysis of BAEE, BAPNA and haemoglobin by partially purified tsetse proteinase inhibitor (0.012 mg protein/ml). 100% activities given as hydrolysis of 0.0099 micromoles of BAEE (□ — □), 0.0013 micromoles of BAPNA (■ — ■) Δ/280 nm for haemoglobin (● — ●), all per minute.

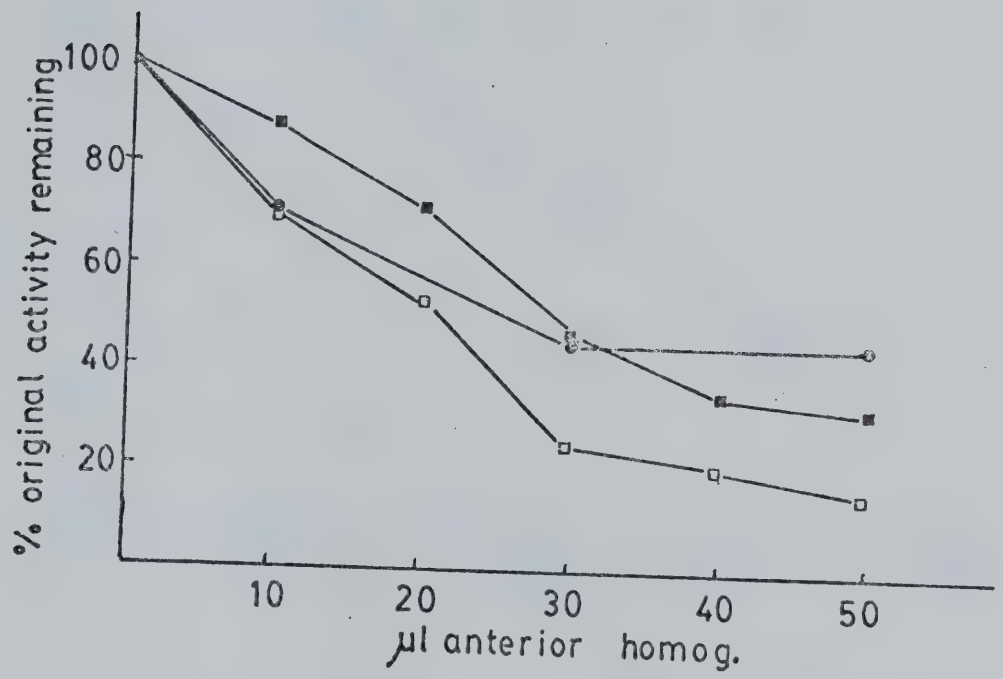

Figure 5b. Inhibition of tsetse trypsin hydrolysis of BAEE, BAPNA and haemoglobin by partially purified tsetse proteinase inhibitor (0.012 mg protein/ml). 100% activities given as hydrolysis of 0.0098 micromoles of BAEE (□ — □), 0.0014 micromoles of BAPNA (■ — ■) Δ A280nm for haemoglobin (● — ●), all per minute.

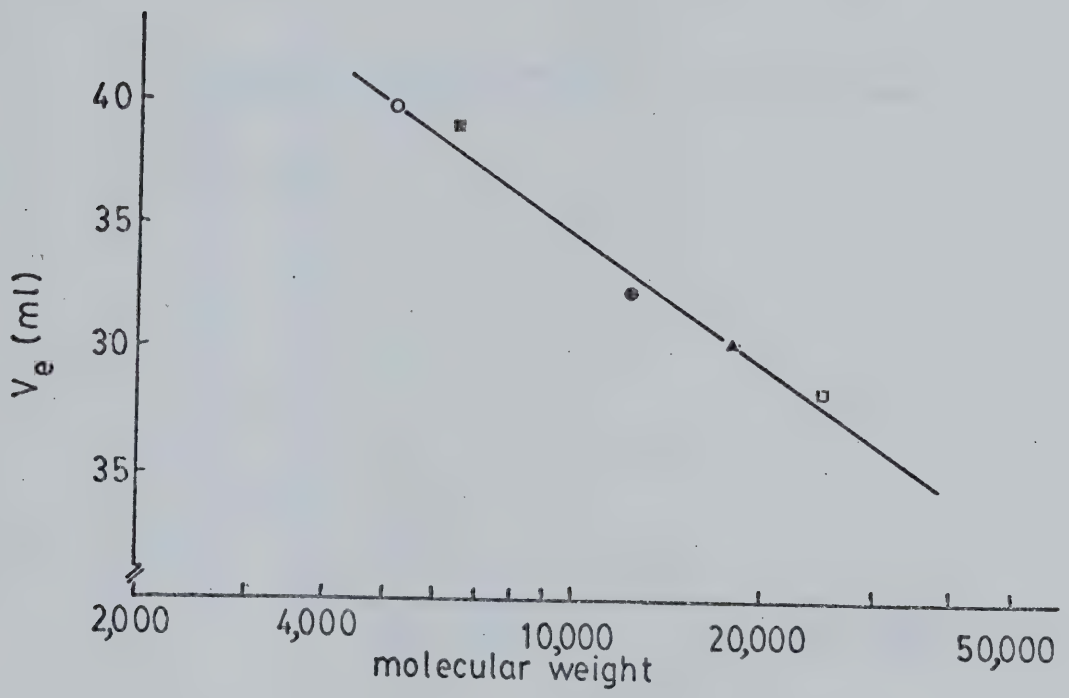

Figure 6. Molecular weight determination of tsetse anterior midgut proteinase inhibitor. Determinations were made using a Sephadex G-50 column calibrated with pancreatic trypsin inhibitor (■), Cytochrome C (●), myoglobin (▲), and trypsin (□). Molecular weight of antiproteinase approximately 5,500 dalton (○). Linear regression was used to fit calibration points to the line.

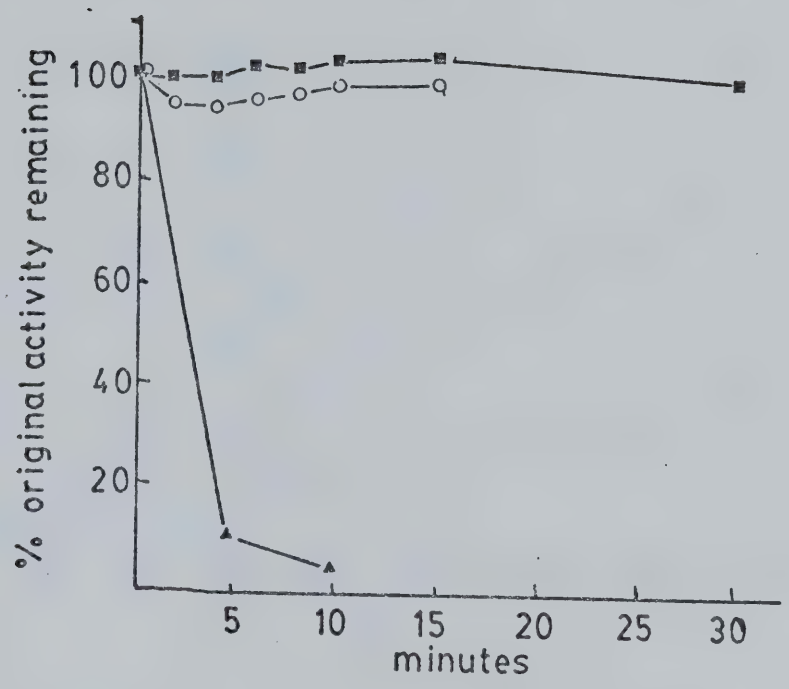

Figure 7. Stability of tsetse proteinase inhibitor to heat, HCl and TCA treatment. Assays were performed by testing the treated material for ability to inhibit hydrolysis of BAPNA by digestive midgut homogenate and compared to untreated material. 100% activities as micromoles substrate hydrolyzed per minute as follows: heat, 80°C (○ – ○) 0.0072 micromoles, HCl, 1 M (■ – ■) 0.0043 micromoles and TCA, 1% (▲ – ▲) 0.0194 micromoles.

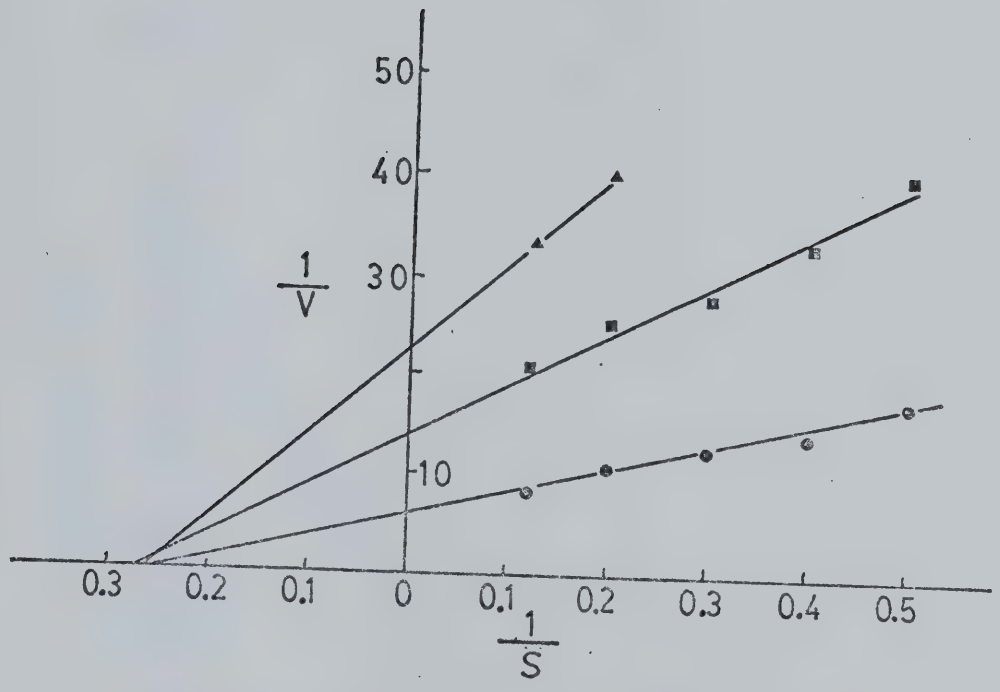

Figure 8. The effect of substrate concentration on trypsin and trypsin inhibitor activity. Trypsin alone (● — ●), trypsin and 0.006 mg antiproteinase, (■ — ■), trypsin and 0.009 mg antiproteinase. (▲ — ▲). S = substrate concentration (mg haemoglobin/ml) and V = reaction velocity (Δ A280 nm per minute).

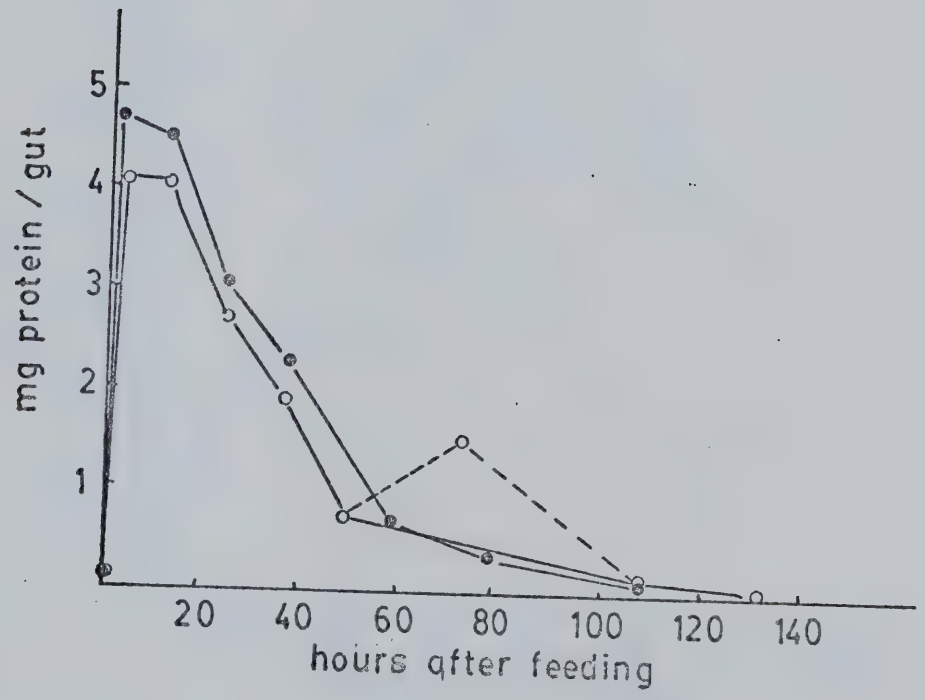

Figure 9a. Protein content of tsetse anterior midgut from teneral (● — ●) and post-teneral (O —O) flies. The insects were starved for three days prior to a single bloodmeal. Samples were collected just prior to feeding and at various times after ingestion of the meal. The point joined by the broken line is the result of a single fly in the sample of 10 that contained a larger quantity of blood in the anterior midgut.

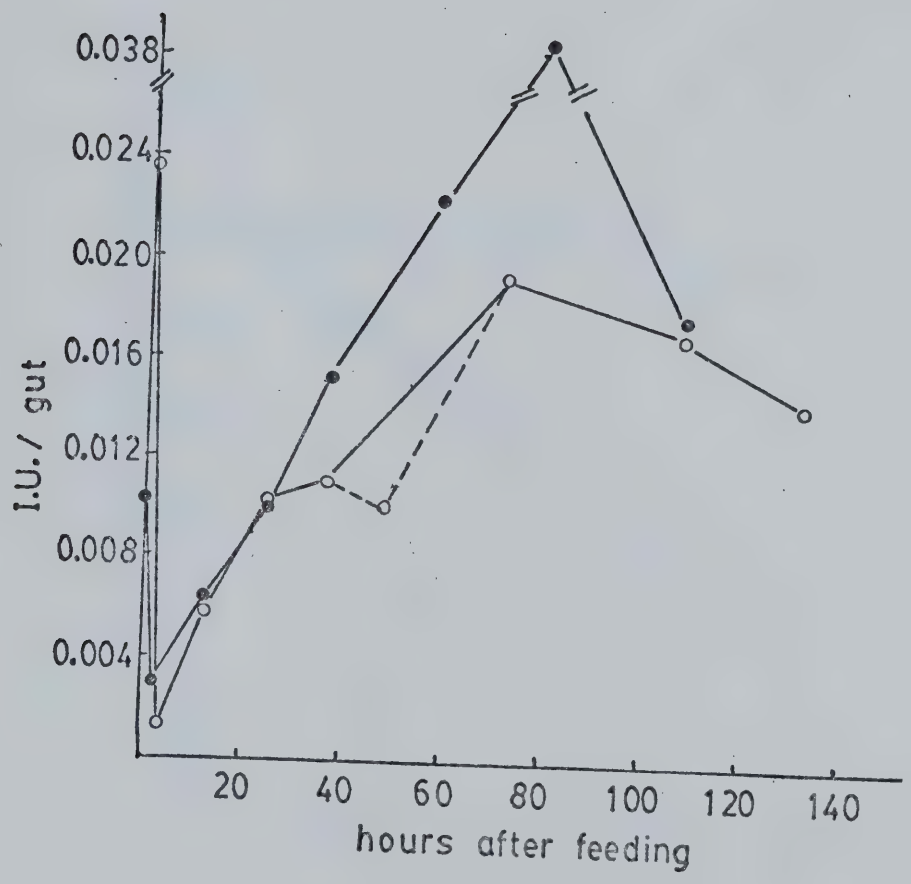

Figure 9b. Tsetse proteinase inhibitor in the anterior midgut from teneral (●—●) and post-teneral flies (O—O). Flies were starved days prior to being a fed a single meal. Samples were collected just prior to feeding and at various times after ingestion of the meal. The point joined by the broken line probably results from deviation from similar age of flies since ingestion of the blood meal.

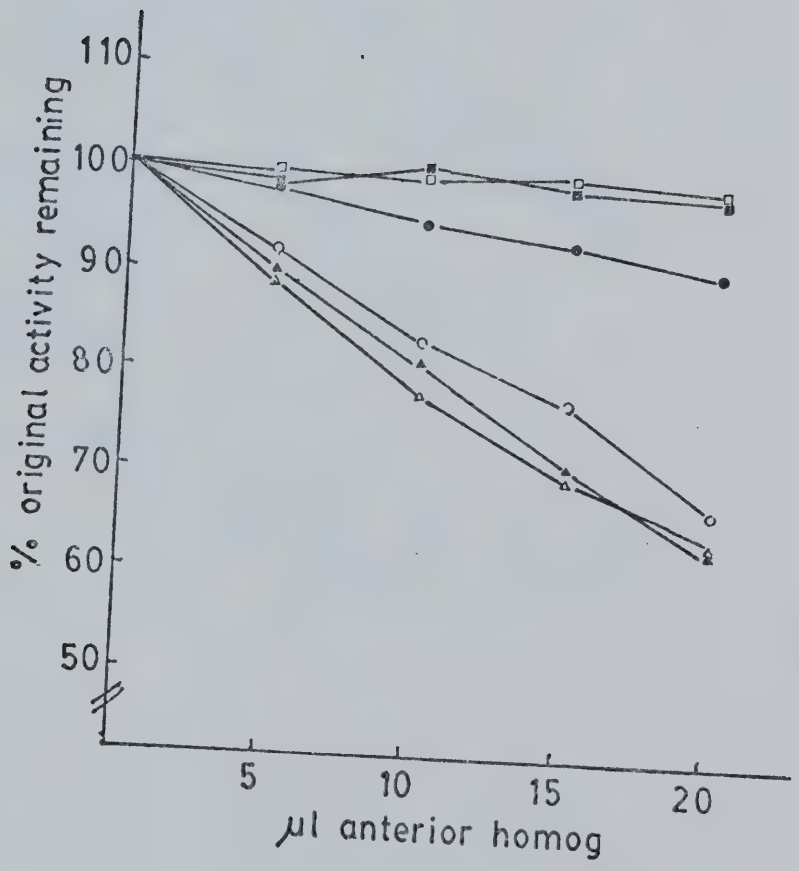

Figure 10. Inhibition of trypsin activity from various sources by partially purified tsetse proteinase inhibitor (0.12 mg/ml protein). Assays were performed by determining the ability of the antiproteinase to inhibit BAPNA hydrolysis by gut homogenate. 100% activities as micromoles of substrate hydrolyzed per minute are given as follows; Bovine trypsin (■—■) 0.0064 micromoles, P. regina (▲—▲) 0.0065 micromoles, G. m. morsitans (△—△) 0.0070 micromoles, A. aegypti (○—○) 0.0072 micromoles, P. americana (●—●) 0.0078 micromoles, and P. adstrictus (□—□) 0.0072 micromoles.

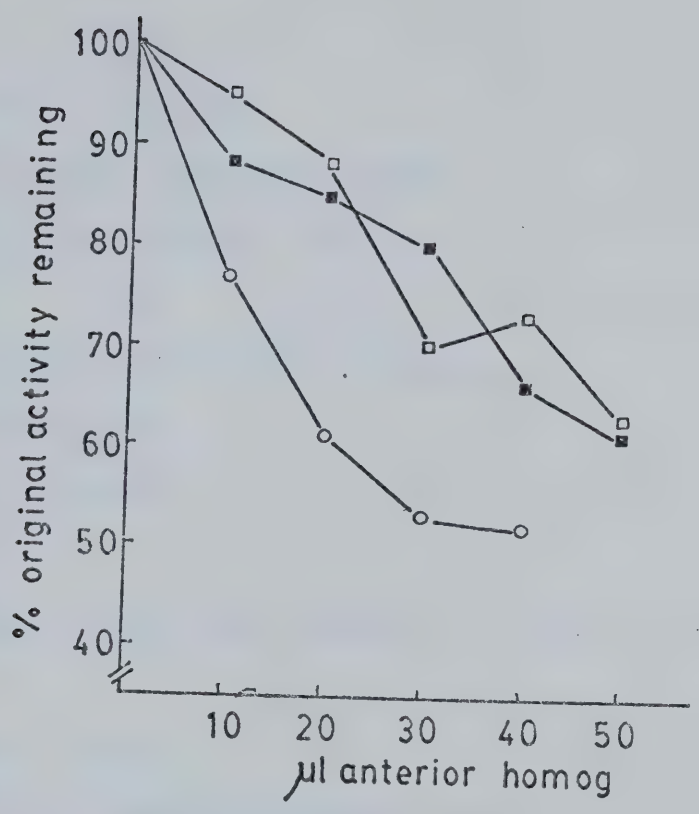

Figure 11. Effect of Rhodnius prolixus crop, anterior midgut, (3.3 mg protein/ml) upon hydrolysis of BANA and casein. 100% activities as ΔA280 nm per minute for casein ΔA520 nm per minute for BANA. Anterior midgut was added to enzyme after thiol activation, casein (○—○) 0.0025 and BANA (■—■)0.033, and before thiol activation with BANA (□—□)0.018.